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Immunohistochemical detection of lipid peroxidation products, protein-bound acrolein and 4-hydroxynonenal protein adducts, in actinic elastosis of photodamaged skin

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Abstract **Acrolein and 4-hydroxy-2-nonenal (HNE) are both byproducts of a lipid peroxidation reaction. Actinic elastosis in photodamaged skin of aged individuals is characterized by the accumulation of fragmented elastic fibers in the sun-exposed areas. To study whether a lipid peroxidation reaction is involved in the accumulation of altered elastic fibers in actinic elastosis, skin specimens obtained from sun-damaged areas were immunohistochemically examined using the antibodies against acrolein and HNE. Both antibodies were found to react with the accumulations of elastic material. Double immunofluorescence labeling demonstrated that acrolein/elastin and HNE/elastin were colocalized in the actinic elastosis. Western blot analysis showed that the polypeptide with a molecular weight of 62 kDa reacted with anti-acrolein, anti-HNE and anti-elastin antibodies. The results suggest that acrolein and HNE may be associated with actinic elastosis.**

Introduction

Two independent, biologically divergent processes occur in cutaneous aging: chronological aging and sun exposure-related aging [1]. Chronic exposure to ultraviolet (UV) radiation causes degenerative alterations in the skin, clinically characterized by wrinkles and atrophy. The major histological changes in actinically damaged skin are the accumulation of basophilic fibers in the upper dermis, referred to as basophilic degeneration or actinic elastosis [1, 2]. Previous histological and biochemical studies on the nature of the accumulated fibers have demonstrated that altered elastin is the primary component of actinic elastosis [3, 4]. The mechanism of the formation of actinic elastosis in photodamaged skin is still unclear. Several investigators have shown that exposure of skin to UV irradiation leads to a sustained elevation of matrix metalloproteinases (MMPs), and various MMPs are associated with the elastotic material of sun-damaged skin [5, 6], implying that degradation of elastic or collagen fibers by MMPs is involved in the formation of actinic elastosis. Mizutani et al. have demonstrated that elastic material in sun-damaged skin react with the monoclonal antibody for N^{ε}-(carboxymethyl) lysine (CML), one of the advanced glycation end products (AGEs) [7], suggesting that glycoxidation of elastic fibers may be involved in the generation of photodamaged skin.

Lipid peroxidation has been demonstrated to cause a wide range of biological effects including atherosclerosis, ischemic heart disease, aging, diabetes mellitus, and cancer [8]. Lipid peroxidation generates undesirable aldehydes that react with various proteins resulting in the formation of byproducts including 4-hydroxy-2-nonenal (HNE). HNE is one of the major α, β-unsaturated aldehydes produced by membrane lipid peroxidation, and can be a potent indicator of lipid peroxidation. Acrolein is an unpleasant and troublesome by-product of overheated organic matter and occurs as a ubiquitous pollutant in the environment, e.g. incomplete combustion of plastic and cigarette smoking. Recently it has been demonstrated that acrolein forms an acrolein-lysine adduct, N^{ε}-(3-formyl-3,4-dehydropiperidino)lysine (FDP-lysine) [9].

It has been demonstrated that the antibodies for HNE and acrolein exhibit a positive reaction with the fibrous cap tissue of atheromatous plaques and foam cells of necrotic tissue in atheromatous lesions [10, 11]. In the present study, we sought to determine whether HNE and acrolein are present in the accumulations of sun-damaged skin using the specific monoclonal antibodies.

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Materials and methods

Skin samples

Normal skin specimens were obtained from the surgical margins of benign skin tumors. Sun-exposed skin samples were taken from the face or neck ($n = 5$) and sun-protected skin samples were taken from the back or buttocks ($n = 5$) of aged individuals (over 55 years old). The degree of elastosis was evaluated using Verhoeff-van Gieson stain. Sun-exposed skin specimens showing tangled, extremely coarse and degenerated elastic fibers accompanied by disorganization into murky, amorphous masses (grade 4) [12] and sun-protected skin specimens without elastotic change (grade 0) were selected for analysis.

Antibodies

Monoclonal antibodies for acrolein-treated keyhole limpet hemocyanin (KLH; clone no. 5F6) [10, 13] and HNE-treated KLH (HNEJ-2) [11, 14] were produced as previously described. The specificity of the antibodies was confirmed by competitive and noncompetitive ELISA as described in the references.

Immunohistochemistry

Normal human skin specimens were fixed in 10% buffered formalin, embedded in paraffin, and cut into 5-μm sections. The sections were pretreated with trypsin (0.25% in phosphate-buffered saline). The sections were soaked in Tris-buffered saline (TBS) containing 5% H_2O_2 for 5 min to inactivate endogenous peroxidase and then incubated with 10% rabbit serum in TBS for 30 min. The sections were incubated with monoclonal anti-acrolein antibody or monoclonal anti-HNE antibody at a dilution of 1:1000 for 24 h. After washing with TBS, the sections were incubated with biotin-conjugated anti-mouse Ig antibody (1:500; Dako, Glostrup, Denmark) for 1 h, washed with TBS, and reacted with peroxidase-labeled avidin-biotin complex (Dako) for 30 min. The reaction was visualized with 3-amino-9-ethylcarbazole. The sections were counterstained with hematoxylin. For double immunofluorescence labeling, skin sections were incubated with anti-acrolein antibody or anti-HNE antibody (1:400) and rabbit polyclonal anti-elastin antibody (1:200) (Elastin Products, Owensville, Mich.). Bound antibodies were visualized with fluorescein-conjugated anti-mouse Ig antibody (Dako) (1:20) and rhodamine-conjugated anti-rabbit Ig antibody (Dako) (1:40). Evaluation of fluorescence was performed with a confocal laser scanning microscope (LSM410; Carl Zeiss, Jena, Germany).

Immunoblot

Samples of photodamaged skin were washed with water at 4 °C exhaustively, then proteins were extracted with 4 *M* guanidine-HCl at 4 °C for 24 h. Insoluble material was pelleted by centrifugation at 10,000 *g* for 30 min. The supernatant was dialyzed against 0.1% acetic acid and lyophilized. The lyophilized samples were dissolved in sample buffer (50 m*M* Tris-HCl, pH 7.4, 1% SDS) and then subjected to 2–15% gradient SDS-PAGE [15]. An aliquot of each sample was taken and the protein content was determined using a protein assay kit (Biorad Laboratories, Hercules, Calif.). The concentration in each sample for SDS-PAGE was adjusted to be equivalent in each well. The samples were transferred from the acrylamide gel to a nitrocellulose membrane (Schleicher & Schuell, Keene, N.H.) [16]. The membranes were incubated with anti-acrolein, anti-HNE, or monoclonal anti-elastin (Elastin Products) antibodies, or with a mixture of anti-acrolein and anti-elastin antibodies at a dilution of 1:1000 for 1 h, then reacted with peroxidase-conjugated anti-mouse Ig antibody at a dilution of 1:2000 for 1 h at room temperature. Antigen-antibody complex was visualized by chemiluminescence (Amersham). To compare the degradation patterns of elastin in photodamaged and sun-protected skin, the proteins extracted from sun-protected skin (buttock) were also analyzed with anti-elastin antibody.

Results

The antibody for acrolein reacted with elastic material and stromal fibroblasts in the papillary dermis in sun-damaged skin (Fig. 1 a). In some skin samples (patients 2 and 4,

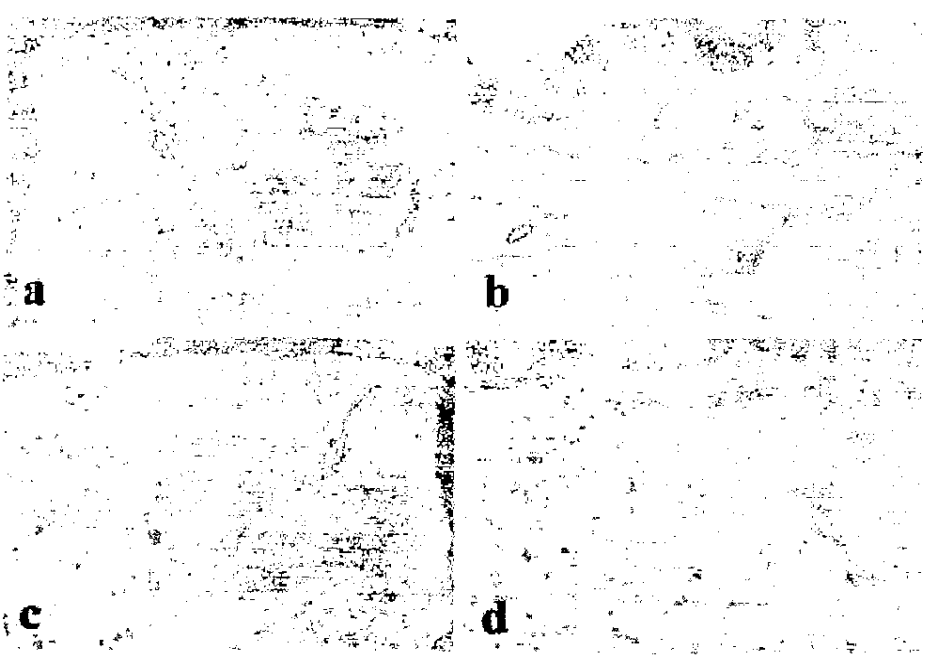

Fig. 1 a–d Immunohistochemistry of photodamaged skin with antibodies for protein-bound acrolein and 4-hydroxy-2-nonenal (HNE). Deparaffinized sections of photodamaged skin (**a, c**) and sun-protected skin (**b, d**) were incubated with monoclonal anti-acrolein antibody (1:1000) (**a, b**) and anti-HNE antibody (1:1000) (**c, d**)

Table 1 Summary of the immunohistochemistry results from sun-damaged and sun-protected skin

Patient no.	Age (years)	Sex	Site	Actinic elastosis[a]	Acrolein	HNE
1	69	M	Face	4+	+	±
2	80	F	Face	4+	±	±
3	72	M	Neck	4+	+	±
4	63	M	Face	4+	±	±
5	71	F	Face	4+	+	±
6	55	M	Back	0	–	–
7	58	M	Back	0	–	–
8	61	F	Back	0	–	–
9	65	F	Buttock	0	–	–
10	55	M	Buttock	0	–	–

[a]The level of actinic elastosis was classified according to the system described in reference 12

Table 1) the intensity of the staining of elastic material was heterogeneous: some areas of the same lesion were positive and some were negative. Fragmented fibers seen just beneath the elastotic material were also positive (not shown). Anti-HNE antibody also reacted with elastotic material but to a lesser extent than anti-acrolein antibody. A heterogeneous staining pattern of the elastotic material which was seen in samples from patients 2 and 4 with anti-HNE antibody was observed in samples from all patients with anti-acrolein antibody (Fig. 1 c). Unlike the antibody for acrolein, anti-HNE antibody did not react with the fragmented fibers present beneath the elastotic material (not shown). Sun-protected skin was not immunoreactive with either antibody (Fig. 1 b, d). The epidermis from sun-exposed and sun-protected areas showed a weak immunoreactivity with both antibodies (Fig. 1 a–d). Skin from a sun-protected area of young (5–16-year-old) individuals ($n = 3$) showed no immunoreactivity with either antibody (not shown). The immunohistochemistry results are summarized in Table 1.

Double immunofluorescence labeling demonstrated colocalization of elastin and acrolein (Fig. 2 a–c). In contrast, anti-HNE antibody was found to recognize only a certain proportion of the elastotic material (Fig. 3 a–c).

In Western blot analysis of skin homogenate isolated from sun-damaged skin anti-elastin antibody detected a major 62-kDa protein and minor 45, 32, 28 and 24-kDa proteins (Fig. 4, lane 1). There were basically no differences between the immunoblot patterns for elastin in sun-damaged and sun-protected skin (Fig. 4, lane 5), suggesting that the degradation of elastin is mainly due to the extraction procedure. The antibody against HNE recognized

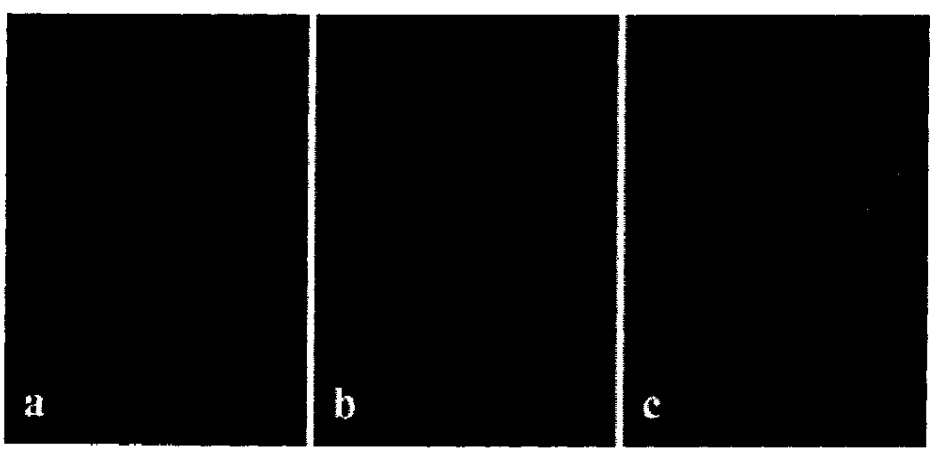

Fig. 2 a–c Double immunofluorescence labeling of photodamaged skin with the antibodies for protein-bound acrolein and elastin. Cryostat sections were incubated with monoclonal anti-acrolein antibody (1 : 400) and rabbit polyclonal anti-elastin antibody (1 : 200). Bound antibodies were visualized with fluorescein-conjugated anti-mouse Ig antibody (1 : 20) (**a**) and rhodamine-conjugated anti-rabbit Ig antibody (1 : 40) (**b**). Evaluation of fluorescence was performed using a confocal laser scanning microscope (**c**)

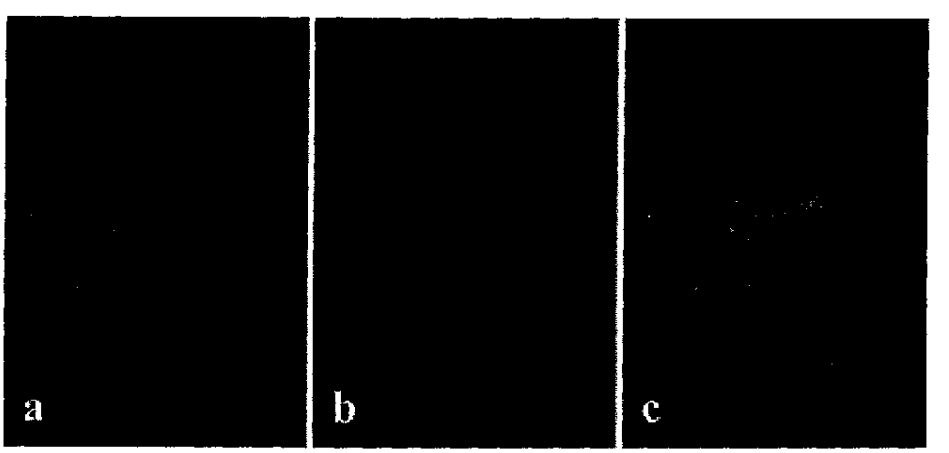

Fig. 3 a–c Double immunofluorescence labeling of elastic material in actinic elastosis with the antibodies for HNE and elastin. Skin sections were incubated with monoclonal anti-HNE antibody (1 : 400) and rabbit polyclonal anti-elastin antibody (1 : 200). Bound antibodies were visualized with fluorescein-conjugated anti-mouse Ig antibody (1 : 20) (**a**) and rhodamine-conjugated anti-rabbit Ig antibody (1 : 40) (**b**). Evaluation of fluorescence was performed using a confocal laser scan microscope (**c**)

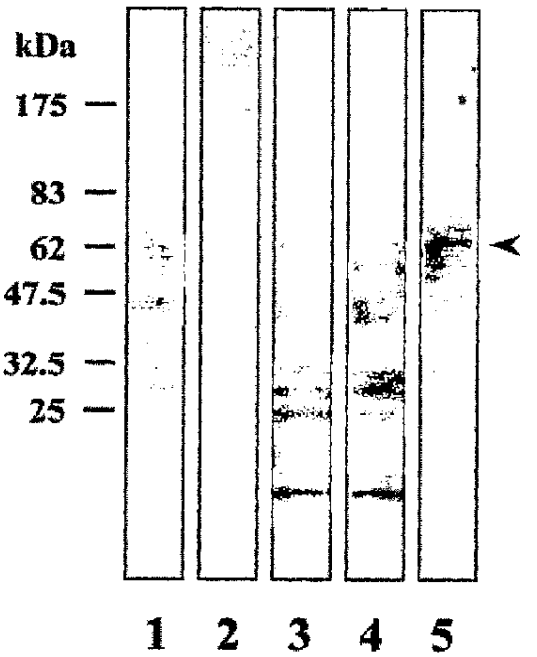

Fig. 4 Western blot analysis of the proteins isolated from photodamaged skin with the antibodies for protein-bound acrolein, HNE and elastin. Skin specimens from sun-damaged areas were washed with water exhaustively. The proteins solubilized by guanidine-HCl were resolved on 2–15% gradient SDS-PAGE. The blots were incubated with anti-elastin antibody (*lane 1*), anti-HNE antibody (*lane 2*), anti-acrolein antibody (*lane 3*), or with a mixture of anti-elastin and anti-acrolein antibodies (*lane 4*) at a dilution of 1 : 1000 for 24 h, then incubated with peroxidase-conjugated anti-mouse Ig antibody at a dilution of 1 : 2000 for 1 h. Antigen-antibody complex was visualized by chemiluminescence. The proteins extracted from sun-protected skin were also analyzed with anti-elastin antibody (*lane 5*). *Arrowheads* indicate 62-kDa polypeptide detected by the antibodies against elastin (*lanes 1 and 5*), HNE (*lane 2*) and acrolein (*lane 3*). Note that the mixture of anti-elastin and anti-acrolein antibodies yielded a single band at 62 kDa (*lane 4*)

major polypeptides with molecular weights of 62, 52 and 48 kDa (Fig. 4, lane 2). Anti-acrolein antibody reacted with major polypeptides with molecular weights of 62, 32, 28, 24 and 16 kDa (Fig. 4, lane 3). The mixture of anti-elastin and anti-acrolein antibodies yielded a single band at 62 kDa, indicating that acrolein is bound to 62-kDa elastin peptide (Fig. 4, lane 4).

Discussion

It has been demonstrated that the elastin in the actinic elastosis of sun-damaged skin is modified by CML, one of the glycoxidation products of AGEs [7]. Recently it has been shown that CML is generated from arachidonic acid without glucose and is considered to be a lipid peroxidation product [17]. This led us to investigate the immunoreactivity of actinic elastosis with antibodies raised against HNE and acrolein. HNE and acrolein were both found in the elastotic material in actinic elastosis, suggesting that these products are associated with actinic elastosis. The significance of the heterogeneous staining for HNE and acrolein in the elastotic material is not clear, but this is likely a secondary phenomenon arising from different degrees of exposure between the two epitopes.

The relationship between HNE and acrolein accumulation and actinic elastosis is uncertain. It has been reported that a physiological concentration of HNE shows growth-modulatory effects on cells via an epidermal growth factor receptor-linked signal pathway [18]. The treatment of macrophages or Kupffer cells isolated from cirrhotic liver with HNE has been found to consistently induce transforming growth factor β1 (TGF-β1) expression [19]. TGF-β1 is considered to be an essential growth factor in development, wound healing and fibrosis [20, 21]. This growth factor induces fibrosis when injected into subcutaneous tissues [22] and stimulates collagen and elastin expression [23, 24]. Although the physiological role of other glycoxidation products and lipoxidation products has not yet been elucidated, HNE and acrolein may be involved at least to a certain extent in the elastotic changes in actinic elastosis via direct modulatory effects on cells or induction of TGF-β1 expression

Since double immunofluorescence labeling of actinic elastosis suggested the colocalization of elastin/HNE and elastin/acrolein, we attempted to identify the target protein by immunoblot assay. Western blotting demonstrated that several polypeptides were immunoreactive with the antibodies for HNE and acrolein. In these polypeptides, only the 62-kDa polypeptide was immunoreactive with anti-elastin antibody. Because the molecular weight of the intact elastin molecule is considered to be 72 kDa and degradation of the elastin molecule occurs in a cassette-like fashion [25, 26], the 62-kDa polypeptide may represent a degradation product of the elastin molecule generated in sun-damaged skin or during the extraction procedure. The latter is more likely because the elastin isolated from sun-protected normal control skin showed a similar degradation product with a molecular weight of 62 kDa.

It has been demonstrated that HNE-treated glucose-6-phosphate dehydrogenase (G6PDH) becomes resistant to proteolysis by the action of multicatalytic proteases [27] resulting in marked accumulation of the protein. In actinic elastosis, HNE-modified elastin may acquire a relative resistance to the degradation by the proteases such as MMPs and may accumulate in the upper dermis. Although enhanced expression of elastin in skin from actinic elastosis has been reported [28], the accumulation of elastic fibers in actinic elastosis may be related to altered degradation as well as elevated expression.

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